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Effects of Prenatal Exposure to Morphine on the Development of Sexual Behavior in Rats

I. U. VATHY,* A. M. ETGEN† AND R. J. BARFIELD†

**Department of Psychology and †Department of Biological Sciences*
Rutgers University, New Brunswick, NJ 08903

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VATHY, I. U., A. M. ETGEN AND R. J. BARFIELD. *Effects of prenatal exposure to morphine on the development of sexual behavior in rats.* PHARMACOL BIOCHEM BEHAV **22**(2) 227–232, 1985.—Females exposed to morphine sulfate *in utero* (5–10 mg/kg twice a day on days 11–18 of gestation) displayed precocious vaginal opening and had increased body weight from the 8th week after weaning. In addition, there was a substantial inhibition in adult feminine sexual behavior. Male rats that received either morphine or saline prenatally did not show any body weight differences, and most of the measures of masculine sexual behavior did not differ between the two groups. However, the male rats exposed to morphine had a significantly shorter post-ejaculatory intromission latency than the saline controls. Examination of cytosol estrogen receptor levels in the hypothalamus-preoptic area (HPOA) of both saline and morphine sulfate-treated female rats revealed essentially identical patterns of depletion and replenishment. Additionally, estrogen treatment was equally effective at inducing HPOA progestin receptor synthesis in both groups. These results show that prenatal morphine treatment at the times and dose level administered disrupts the development of reproductive function in females but has only minor effects on male reproductive function.

Morphine Development Reproductive behavior Steroid receptors

A series of developmental events that correlate with the onset of central nervous system (CNS) susceptibility to hormones involved in sexual differentiation occurs during mid- to late gestation in rats. These changes include: (1) final cell division of hypothalamus and preoptic area neurons between days 14–17 [11]; (2) the appearance of steroid receptors in the hypothalamus and preoptic area around day 15 [19]; and (3) the onset of gonadal steroid secretion at approximately the same time [14]. Any or all of these may serve as signals for the onset of the "critical period" of brain sexual differentiation [18].

In addition to the emergence of steroid receptors, opiate receptors begin to appear in the brain on the 14th day of fetal life or perhaps even earlier [1]. Hence, the time of appearance of opiate receptors closely parallels the timing of other developmental events which correlate with the sensitive period for brain sexual differentiation.

Recently we demonstrated [17] that adult reproductive function of female rats can be affected by prenatal exposure to morphine sulfate during the period of organogenesis (5–14 days of gestation). The period of organogenesis, however, occurs much earlier than hormone dependent brain sexual differentiation. It seemed possible that the processes of feminization and/or masculinization of reproductive behavior might be influenced further by prenatal morphine sulfate exposure just prior to and during the period of brain sexual differentiation. Therefore, the present study examined the effects of chronic exposure to morphine during mid- to late gestation on the development of male and female sexual behavior in rats.

METHOD

Materials

Progesterone (P), 17β-estradiol (E_2) and 17β-estradiol benzoate (E_2B) were obtained from Steraloids, Inc. (Wilton, NH). Diethylstilbestrol (DES) was purchased from Sigma Chemical Co. (St. Louis, MO). The radiolabeled hormones [(2,4,6,7-^{3}H)-E_2, spec. act. 90–110 Ci/mmol; 17α-(methyl-^{3}H)R5020, spec. act. 87 Ci/mmol] and unlabeled R5020 were purchased from New England Nuclear Corp. (Boston, MA) and checked for purity by thin layer chromatography before use. All other chemicals were of reagent grade.

General Method

Pregnant female Sprague-Dawley rats were obtained from Blue Spruce Farms, Inc. (Altamont, NY) on the fifth day after conception. (The day of conception was counted as day zero.) Upon arrival all animals were weighed and randomly assigned to one of two groups: (1) a morphine sulfate (MS) treated experimental group; or (2) a saline (S) treated control group. They were then housed individually in maternity cages (47×25×15 cm) with food and water available ad lib and were maintained on a 12-12 hour reverse light-dark cycle with lights off at 0930 hours.

On day 11 post-conception the experimental group was injected twice, once in the morning (0800 hours) and once in the evening (2000 hours) with 5 mg/kg MS subcutaneously (SC). On day 12 the animals were injected with 5 mg/kg MS in the morning and 10 mg/kg in the evening. On day 13 and thereafter through day 18 the animals received two daily injections of 10 mg/kg MS. The control group of female rats received two daily injections of 0.8% NaCl on day 11 to 18. These injection times were chosen to coincide with the appearance of androgen, estrogen [19] and opiate [3] receptors in the brain.

On the day of birth pups were sexed and weighed, then each litter was adjusted to 10 (6 females and 4 males). All dams had more than 6 females and 4 males except three which were eliminated at this point. The offspring of MS treated mothers were tattooed on both front foot-pads with black ink. Then each of the MS treated mothers with her pups was randomly paired with one of the S treated mothers and her pups. The litters were divided in half (3 females and 2 males) and cross-fostered such that half of the MS treated pups were given to the S mothers and half of the S treated pups were given to the MS mothers. Hence, each mother (MS or S) raised half of her own litter and half of the adopted ones. After cross-fostering pups were left undisturbed until weaning at day 21. At weaning all pups were weighed, ear-punched for identification, housed individually in standard rat cages (30×18×18 cm) and maintained on a 12-12 hour reversed light-dark cycle (lights off at 0930 hours) with food and water available ad lib. All animals were weighed once a week throughout the entire experiment. The female rats were checked daily for vaginal opening beginning on the 25th day. Vaginal smears were taken for a period of two weeks (from the 45th day) to determine estrous cyclicity.

Behavioral Testing

Females. At 60 days of age the animals were ovariectomized under Metofane anesthesia (Pitman-Moore, Inc., Washington Crossing, NJ). Four days after ovariectomy behavioral testing began and continued once a week for six consecutive weeks. Female rats were tested with sexually experienced male rats in glass walled observation tanks measuring 51×25×30 cm with wood shavings on the floor. Female rats received SC injections of one of three doses (2 μg, 8 μg or 25 μg) of E_2B and 500 μg of P 54 and 5 hours, respectively, prior to each test. All female rats were tested twice with each dose of E_2B in a random order. Stimulus male rats were allowed to adapt to the test chamber for at least ten minutes prior to the introduction of the female. Males were permitted to mount female rats ten times, and the number of lordosis responses was recorded. A lordosis quotient (LQ, number of lordosis/number of mounts × 100) was derived and served as a measure of estrous responsiveness. Solicitation behavior including darting, hopping, and ear-wiggling was recorded whenever it occurred throughout the 10-mount test. In addition, the quality of lordosis (a subjective estimate of lordosis posture) was scored according to the scale of Hardy and DeBold [10]: (1) a shallow arching of the back, (2) definite dorsiflexion of the spine, (3) an exaggerated lordosis posture.

Males. From 75 days of age the gonadally intact male rats were tested for masculine sexual behavior with stimulus female rats at weekly intervals for five consecutive weeks. The occurrence of mounts, intromissions and ejaculations was recorded on an event recorder. In addition, an ultrasonic microphone connected to a Holgate bat detector monitored the 22 kHz ultrasonic vocalization emitted by the males during the post-ejaculatory interval. The following measures were derived from the raw data: Mount Latency (ML, the time from the introduction of the female to the first mount), Intromission Latency (IL, the time from the introduction of the female to the first intromission), Ejaculation Latency (EL, the time from the first intromission to ejaculation), Vocalization Latency (VL, the time from ejaculation to the beginning of the 22 kHz vocalization), Vocalization Duration (VD, the time from ejaculation until the end of the 22 kHz vocalization), Post-Ejaculatory Intromission Latency (PEIL, the time from ejaculation until the first intromission of the next copulatory series). The testing period was terminated after 30 minutes without a mount or intromission or until the end of the first post-ejaculatory interval.

For each measure, means were calculated for each subject based on his/her performance in the weekly tests of male/female sexual behavior. All behavioral measures were subjected to analysis of variance, and Duncan's multiple range test was used for post-hoc pairwise comparisons [7].

Steroid Receptor Assays

At least two weeks after the final behavior tests, steroid hormone receptor levels were determined in the hypothalamus-preoptic area (HPOA) of both MS and S animals. Two laboratories [12,22] recently presented evidence that steroid binding sites which have traditionally been considered cytoplasmic (i.e., cytosol receptors) may be of nuclear origin. For the purposes of this report, we will use the term "cytosol receptor" to refer to those binding sites which are available for steroid binding following homogenization and centrifugation regardless of their subcellular origin. Likewise, "depletion" may refer to high affinity binding of activated (i.e., steroid-bound) receptors within the nucleus rather than actual depletion of receptors from the cytoplasm. To assess cytosol estrogen receptor depletion and replenishment, rats received SC injections of 8 μg of E_2B and were sacrificed 0, 1, 2, 4, 12 or 24 hours later. Estrogen induction of cytosol progestin receptors was measured 48 hours after injection of oil vehicle or 8 μg of E_2B. Animals were sacrificed by decapitation, the HPOA dissected as described previously [8], and steroid receptors assayed using a modification of the method of Ginsburg *et al.* [9]. For estrogen receptor measurements, pooled tissue samples from two MS or S animals were homogenized in ice-cold buffer (10 mM Tris-HCl, pH 7.4 at 0°C; 1.5 mM Na_2EDTA; 1.0 mM dithiothreitol; 20% (v/v) glycerol; TEDG) in Teflon-glass tissue grinders and the homogenate centrifuged for 10 min at 850 × g. The resulting supernatant was recentrifuged at 105,000 × g for 1 hr and the clear high speed supernatant (cytosol) removed and used immediately. Aliquots (150 μl) of cytosol were incubated for 10 min at 30°C with 2 nM ^3H-E_2 with or without a 1,000-fold excess of unlabeled DES. Bound ^3H-E_2 was separated from free ^3H-E_2 after a 30 min incubation on Sephadex LH-20 columns equilibrated in TEDG and maintained at 2°C. Bound radioactivity eluted in the first 0.8 ml following the void volume and was collected directly into scintillation minivials. Scintillation fluid (5 ml Tritosol) was added, and radioactivity was counted in a Beckman LS-100C liquid scintillation counter (<2% error) with an automatic external standard. Data were converted to disintegrations per min/mg cytosol protein. Specific binding was calculated by subtracting nonspecific binding (with excess DES) from total binding (without DES).

TABLE 1

FREQUENCY AND CUMULATIVE DISTRIBUTION OF DATES OF VAGINAL OPENINGS AS A FUNCTION OF AGE IN BOTH GROUPS

Treatment	Distribution	Age (days) 29	30	31	32	33	34	35	36	37	38	39	N
MS	Frequency	1	4	9	5	4	7	3	2				35
	Cumulative		5	14*	19	23	30	33	35				
S	Frequency			1	10	8	7	2		1		1	30
	Cumulative				11	19	26	28		29		30	

*Significant difference between cumulative distribution. $D_{max}=0.367$, $p<0.04$.

TABLE 2

THE EFFECTS OF PRENATAL MORPHINE SULFATE ON FEMALE SEXUAL BEHAVIOR

EB Dose	Experimental Treatment*	N	Behavioral Measures LQ	QL	SS
2 μg	MS	32	38.19 ± 6.40	0.63 ± 0.12	2.50 ± 0.48
	S	30	76.33 ± 5.33	1.61 ± 0.13	6.05 ± 0.70
8 μg	MS	32	38.59 ± 6.00	0.67 ± 0.11	3.02 ± 0.59
	S	30	74.47 ± 4.64	1.63 ± 0.13	6.70 ± 0.68
25 μg	MS	32	46.91 ± 6.25	0.94 ± 0.13	4.13 ± 0.73
	S	30	79.50 ± 4.57	1.78 ± 0.12	7.43 ± 0.69

Note: Values are expressed as mean ± SEM.
(LQ=lordosis quotient; QL=quality of lordosis; SS=solicitation scores).
*Significant overall treatment effect (p's<0.001) for all measures.

Cytosol progestin receptors were quantified in a one-point assay using a subsaturating concentration of ^{3}H-R5020 to reduce interference from high affinity, non-specific binding [2]. Details of the assay were the same as for estrogen receptors with the following modifications. In all steps dithiothreitol in the buffer was replaced with 12 mM monothioglycerol. Cytosols were incubated for 4 hr at 0°C with 0.5 nM ^{3}H-R5020 in the presence or absence of 1,000-fold excess unlabeled R5020, and samples were not incubated for 30 min on Sephadex LH-20 columns prior to elution of bound radioactivity.

RESULTS

Analyses of variance revealed that neither female nor male rats prenatally treated with S or MS showed significant differences on any measure which could be attributed to maternal care. Therefore all data analyses reported used combined data from MS or S rats raised by both MS and S mothers.

The weights of the MS and S animals at birth and at weaning were not different for either sex. This trend continued in the male rats throughout the entire experiment. However, the body weights of the MS treated female rats started to accelerate at the 8th week after weaning (MS=210.3 g vs. S=201.2 g), and MS females continued to weigh more than S females until the end of the experiment (MS=258.7 g vs. S=240.2 g). MS females were significantly heavier, $F(1,63)=4.24$, $p<0.05$, they gained more weight each week, $F(13,751)=962.3$, $p<0.001$, and they gained faster over time, $F(13,751)=2.96$, $p<0.001$, than the S controls.

The vaginal openings of the S treated group took place between 31–39 days of age, whereas in the MS group the vaginal openings occurred earlier, between 29–36 days of age (Table 1). The difference between the cumulative distribution of the two treatment groups was significant by a Chi-square approximation of Kolmogorov-Smirnov two-sample test ($D_{max}=0.367$, $\chi_2=8.702$, $p<0.04$, two tailed; [15]). However, as indicated by the daily vaginal smears, both the prenatally MS and S treated females had regular estrous cycles (data not shown).

Behavioral Measures

In all behavioral measures the S treated female rats scored higher than the MS treated females regardless of E_2B dose (2 μg, 8 μg, or 25 μg; see Table 2). The prenatal exposure to MS significantly reduced the frequency of lordosis responses, $F(1,58)=27.84$, $p<0.001$, and the quality of each lordosis, $F(1,58)=38.53$, $p<0.001$, that the animals exhibited. The solicitation scores were also significantly decreased in the MS treated female rats, $F(1,58)=21.72$, $p<0.001$, when compared to controls.

On the contrary, the MS treated male rats were not significantly different from the S treated males on any behavioral measure prior to ejaculation (ML, IL and EL; Table 3). In

TABLE 3

THE EFFECTS OF PRENATAL MORPHINE SULFATE ON MALE SEXUAL BEHAVIOR

Experimental treatment	N	Behavioral Measures ML	IL	EL	VL	VD	PEIL
MS	19	75.5 ± 15.9	92.66 ± 18.1	990.1 ± 152.2	55.04 ± 7.4	179.4 ± 18.6	285.5 ± 23.5*
S	10	119.1 ± 28.9	249.7 ± 85.3	1001.5 ± 134.5	65.4 ± 5.3	202.0 ± 20.2	357.3 ± 9.7

Note: Values are expressed as means ± SEM.
*$p<0.02$.

TABLE 4

DEPLETION OF HPOA CYTOSOL ESTROGEN RECEPTORS AFTER A SINGLE INJECTION OF 8 μg OF E_2B

Prenatal Treatment	Post-injection time, hr:	Specifically Bound ^{3}H-E_2 (dpm/mg protein) 0	1	2	4	12	24
S		3675	*2450	*1900	1290	235	845
MS		3270	2550	*2400	1650	665	1430

Each value represents the mean of two independent replications.
*Data from a single determination.

addition, no differences were detected between the two groups in VL and in VD of the 22 kHz calls emitted after ejaculations. However, there was a significant reduction in the PEIL of the prenatally MS exposed animals, $F(1,14)=8.35$, $p<0.02$, when compared to S controls.

Steroid Receptor Dynamics

Under the described assay conditions, noninjected S and MS females showed similar levels of HPOA cytosol estrogen receptors (S=3675 dpm/mg protein; MS=3270 dpm/mg protein; Table 4). Table 4 also shows the magnitude and time course of estrogen receptor depletion and replenishment in S and MS rats during the first 24 hr following SC injections of 8 μg of E_2B. Although S animals showed slightly greater receptor depletion at each time point between 1 and 12 hr and less replenishment at 24 hr post-injection, the pattern of depletion and replenishment was essentially identical for the two groups. Receptors were depleted by 20–30% in both groups after 1 hr and were at their lowest level (20% of control for MS and 6% for S) at 12 hr post injection. Both MS and S animals also showed significant replenishment by 24 hr post-injection. It is also apparent (see Table 5) that the same E_2B treatment was equally effective at inducing HPOA progestin receptor synthesis in S and MS rats. Treatment for 48 hr with 8 μg of E_2B produced 111% elevations in HPOA cytosol progestin receptors in both groups.

TABLE 5

INDUCTION OF HPOA CYTOSOL PROGESTIN RECEPTORS 48 HR AFTER 8 μg OF E_2B OR OIL VEHICLE

Prenatal treatment	Presacrifice treatment:	Specifically bound ^{3}H-R5020 (cpm/mg protein) Oil	EB	% induction
S		360	760	111
MS		380	800	111

Each oil value represents a single determination, and each EB value represents the mean of two independent replications.

DISCUSSION

The results of the present experiment extend our previous findings [17] that prenatal exposure to MS causes a significant inhibition in adult sexual behavior of female rats. Receptive behavior was reduced as shown by the decrement in the frequency and the quality of lordosis, and proceptivity (measured by solicitation score) was also decreased when compared to control females. The magnitude of the behavioral inhibition by the prenatal MS treatment was greater than in the previous study. In the present experiment, MS females showed an approximately 40% decrease on each behavioral measure as opposed to our earlier findings of 15–20% reduction.

Despite the significant deficit in female sexual behavior, our results indicate that *in utero* exposure of male rats to 10–20 mg/kg of MS on days 11 to 18 of gestation resulted in no such effects. In contrast, MS-treated males exhibited normal levels of copulatory behavior and a significantly shorter PEIL than controls. The results presented here appear to differ from the findings of demasculinized behavioral tendencies by Ward et al. [20]. In the latter authors' study MS was administered on days 15–22 of gestation; therefore, the MS injections corresponded in time with the prenatal

testosterone surge believed to be necessary for behavioral masculinization [21]. In addition, Ward *et al.* [20] used a higher dose of morphine and administered it every 8 hours rather than every 12 hours. These procedural differences may account for the apparent discrepancy between the two studies. Whether or not the shorter PEIL and the otherwise normal copulatory activity are true indications of prenatal MS effects on developing masculine sexual behavior is unclear.

In addition to the behavioral deficits in females, vaginal openings in the MS group occurred earlier than in the control animals. Vaginal opening had occurred in 40% of the MS treated female rats before it was detected in the first S female. The major portion of the vaginal openings in the control group occurred over a very narrow time range (32–34 days of age), comparable to our previous result [17]. Despite precocious vaginal opening in MS rats, daily vaginal smears revealed that normal estrous cycles of 4–5 days occurred in both MS and S treated females.

Prenatal administration of MS did not decrease litter size or body weight. In fact, increased body weight was observed in adult MS treated female rats, consistent with our previous work [17]. However, in the present study the body weights of the MS female rats began to differ significantly from controls only from the 8th week after weaning.

The findings of the present study closely parallel our previously observed changes in body weight, vaginal opening and reproductive behavior in prenatally MS-exposed female rats. Moreover, animals in the present study were cross-fostered at birth whereas those in the previous experiment were not. Thus, the measured differences cannot be explained by an indirect effect of prenatal MS on postnatal maternal factors.

A possible explanation for the behavioral deficit may be that prenatal exposure to MS induced alterations in liver metabolism of steroids. If so, MS animals may metabolize estrogens more rapidly, resulting in reduced access of E_2 to the brain. We tested this hypothesis by measuring the depletion and replenishment of HPOA cytosol estrogen receptors. The data showed slightly less depletion in MS than in S rats at each time after a single injection of 8 μg of E_2B. This may be explained by the fact that the body weight of the MS females was greater than that of the S females. However, the pattern of depletion and replenishment of estrogen receptors was essentially identical, indicating that there was similar access of E_2 to the brain in both groups. Moreover, the induction of HPOA cytosol progestin receptors 48 hr after the E_2B injection was also identical in both groups of females. These results suggest that the estrogen was indeed transported into brain cell nuclei and that some physiological responses (e.g., progestin receptor induction) were triggered.

Another explanation for the behavioral deficit is that MS increased or decreased the sensitivity of opiate receptors in different areas of the brain, e.g., in the limbic structures or the hypothalamus and medial thalamus (neural centers receiving nociceptive input). Perhaps the prenatal treatment altered the perception or awareness of pain, or the emotional responses to pain in the MS rats during intromission. It is known that cervical stimulation elevates pain threshold, induces sexual receptivity and prolongs lordosis responsiveness in rats [4,5].

Alternatively, the possibility that MS caused a reduction in the number of opiate or other neurotransmitter receptors in the spinal cord [13], which may affect the sensitivity of sensory nerves in the flanks, should also be considered. Changes at the spinal cord level may have caused the MS females to perceive the copulatory stimuli as insufficient to produce a lordosis reflex. It is also possible that prenatal MS treatment affects brain structures involved with reproductive functions. It has been shown that morphine accumulates in the cell nuclei of developing CNS neurons [16].

Finally, there is a possibility that the prenatal MS treatment used in this study affected in some way the functional interaction between steroid hormones and brain neurotransmitters. Drug-induced changes in specific neurotransmitter systems, particularly aminergic neurotransmitters, have been shown to alter both the form and intensity of steroid effects on the neural regulation of anterior pituitary function and on behavior, including reproductive behavior [1,6]. The effects of the prenatal MS on neurotransmitters are likely to be diverse and complex; to suggest a more specific mechanism based on our results would be highly speculative.

Our results suggest that the development of feminine and masculine reproductive behavior can be affected by *in utero* exposure to morphine sulfate. Opiate exposure just prior to brain sexual differentiation can defeminize female sexual behavior and somewhat enhance masculine sexual behavior. It is also clear that the behavioral deficits are not the result of the failure of neural estrogen receptors to be translocated or to induce progestin receptor synthesis. However, more studies are necessary to determine whether the disruptive effect of prenatal morphine on the activation of reproductive behavior is through a hormonal or a non-hormonal mode of action during fetal development.

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